PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITIES OF ETHANOLIC EXTRACT OF CALOTROPIS PROCERA FLOWERS AGAINST HUMAN PATHOGENIC STRAINS

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ABSTRACT

Ethanolic extract of flowers of Calotropis procera R. Br. (Asclepiadaceae) shows antibacterial action against four Gram positive micro-organisms, (Staphylococcus aureus, Bacillus subtilis, Bacillus pumilis, and Micrococcus lutes) and Gram Negative micro-organism (Escherichia coli Pseudomonas aeruginosa, and Proteus vulgaris). The antibacterial action of extract is compared with a standard drug (rifampicin, 50μg/ml) at various concentrations (5, 10, 20, 30 and 50 μg/ml). Before that preliminary phytochemical screening was performed by using a standard chemical test and antibacterial action screened by using Cylinder-plate assay method. Phytochemical analysis of the flowers extract showed the presence of tannins, steroids, saponins and flavonoids while alkaloids are absent. Phytochemical investigations explore active constituents which are very significant in drug development. The study revealed a notable antibacterial inhibitory activity of ethanolic extract of the flowers.

Keywords: Calotropis procera, Preliminary phytochemical screening, Cylinder-plate assay method.

INTRODUCTION

Calotropis procera R. Br. (Asclepiadaceae) is a plant widely distributed in Asia, Africa, and Northeast of Brazil. The plant is popularly known because it produces large quantity of latex, which is easily collected from its green parts, when the plant is wounded (Larhnni et al. 1997, Haque et al., 2000). The milky latex is locally applied in the treatment of cutaneous diseases such as ringworm, syphilitic sores and leprosy (Kew, 1985). In Ayurveda: the Indian system of medicine, this plant is reported for the treatment of several infectious diseases including purulent wound infections (Sharangdhar Samhita, 1964, Charaka Samhita, 1952, Sushruta Samhita, 1955). The plant has been widely used in the traditional system of medicine for the treatment of various ailments. It has been used as a purgative, antihelminthic, digestive, stomachic, emetic, expectorant, sedative, an antidiote for snake poisoning and for the treatment of ulcers, tumors, leprosy, asthma, boils, dysentery, eczema, piles, diseases of liver, and spleen disorders (Kirtikar and Basu, 1935, Nadkarni and Nadkarni, 1960). This study showed preliminary phytochemical screening and antibacterial activities of Calotropis procera flowers extract against human pathogenic strains.

MATERIALS AND METHODS

Plant Material

The flowers are collected in month of September-October from out fields of Agiripalli Mandal, Krishna district, identified and herbarium specimen was deposited in the department of Pharmacognosy with specimen No.NRI/OL/P.CDG/1/F (Flowers). It was air dried under shade at ambient temperatures and grounded to small. The plant was first identified at the field using standard keys and descriptions (Gill, 1987).

Preparation of extracts

The shade-dried powder of flowers was subjected to extraction in soxhlet extractor with 70% EtOH for 70 hours (extract yield: 9%) and extract is collected. The collected extract is evaporated to dryness and stored at 4°C until used.

PHYTOCHEMICAL SCREENING

Test for reducing sugars

One gram of the ethanolic extract was weighed and placed into a test tube. This was diluted using 10 ml of de-ionised distilled water. This was followed by the addition of Fehling's solution. The mixture warmed to 40°C in water bath. Development of brick-red precipitate at the bottom of the test tube was indicative of the presence of a reducing sugar (Brain and Turner, 1975).

Test for protein

Millions test, Crude ethanolic extract was mixed with 2 ml of Millions reagent (mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appeared, which turned red upon gentle heating. Ninhydrin test, Crude extract when boiled with 0.2 % solution of Ninhydrin, violet color appeared, indicates presence of amino acids and protein (Debela, 2002).

Test for fat

Stain test, the small quantity of crude ethanolic extract was pressed between two filter papers; the stain on 1st filter paper indicated the presence of fixed oils. Saponification test, In small quantity of crude extract few drop of 0.5N of alcoholic potassium hydrogen were added to which a drop of phenolphthalein was added separately and heated in a water bath for 1 hour. The formation of soap indicated the presence of fixed oils and fats (Debela, 2002).

Test for resins

Two grams of the ethanolic extract was dissolved in 10ml of acetic anhydride. A drop of concentrated sulphuric acid was added. Appearance of purple colour, which rapidly changed to violet, was indicative of the presence of resins. Same procedure was repeated using the aqueous extract of the plant material (Cuilel, 1994).

Test for tannins

Two grams of the ethanolic extract was weighed and placed in a test tube. Two drops of 5% ferric chloride solution was then added. The appearance of a dark green color was indicative of the presence of tannins. The same procedure was repeated using the ethanolic extract (Cuilel, 1994).

Test for steroids

One gram of the ethanolic extract was weighed and placed in a test tube. This was dissolved in 2 ml of acetic anhydride, followed by the addition of 4 drops of chloroform. Two drops of concentrated sulphuric acid were then added by means of a pipette at the side of the test tube. The development of a brownish ring at the interface of the two liquids and the appearance of violet color in the supernatant layer were indicative of the presence of steroid
glycosides. Same procedure was repeated using the aqueous extract (Cuilel, 1994).

**Test for flavonoids**

Two grams of the ethanolic extract was weighed, placed in a test tube, followed by the addition of 10 ml of DMSO. The mixture was heated, followed by the addition of magnesium metal and 6 drops of concentrated hydrochloric acid. The appearance of red colour was indicative of the presence of flavonoids. Same procedure was repeated using aqueous extract (Sofowora, 1993).

**Test for alkaloids**

One gram each of the ethanolic extract was weighed and placed into two separate test tubes. To the first test tube, 2-3 drops of Dragendorff's reagent was added while 2-3 drops of Meyer's reagent were added to the second test tube. The development of an orange-red precipitate (turbidity) in the first test tube (with Dragendorff's reagent) or white precipitate (turbidity) in the second test tube (with Meyer's reagent) was indicative of the presence of alkaloids (Cuilel, 1994).

**Test for saponins**

Five grams of the ethanolic extract was weighed and placed in a test tube. This was followed by the addition of 5 ml de-ionised distilled water. The content was vigorously shaken. The appearance of a persistent froth that lasted for 15 minutes was indicative of the presence of saponins (Brain and Turner, 1975). Table 1 shows the presence of phytochemical characteristics of ethanolic extract flowers.

**ANTIBACTERIAL ACTIVITY**

**Test organisms**

The selected NCIM (National Collection of Industrial Micro-organisms) type bacterial strains were provided by Department of Pharmaceutical Biotechnology, Hindu College of Pharmacy, Amaravathi Road, Guntur. The selected micro-organism was listed below: Four Gram positive micro-organisms (Staphylococcus aureus NCIM 2079, Bacillus subtilis NCIM 2063, Bacillus pumulis NCIM 2327, and Micrococcus lutes NCIM 2871) and Gram Negative micro-organism (Escherichia coli NCIM 2027, Pseudomonas aeruginosa NCIM 2037, and Proteus vulgaris NCIM, 2027).

**Preparation of inoculums**

The in vitro screening of antibacterial activity was carried out using Cylinder-plate assay method. For antibacterial activity, the inoculums or microbial suspension is prepared according to the procedure given in the IP (Indian pharmacopoeia-2010). The test organism (one loop full) were seeded to the nutrient agar (HIMEDIA) at temperature between 40° and 50° and immediately pour the inoculated medium into the Petri plate (8 inch) to give a depth of 3 to 4 mm and allowed to solidify and punched with a sterile cork borer (6.0 mm diameter) to make open cavities. Each plate had maximum seven cavities with appropriate distances.

**Preparation of test and standard solutions**

The stock solution of test sample was prepared by dissolving the dried ethanolic extracts of flowers of *Calotropis procera* at concentration of 5,10,20,30 and 50 μg/ml in dimethylsulphoxide (DMSO) respectively. The stock solution of reference standards (Rifampicin) was prepared at a concentration of 1mg/ml in DMSO. The 50μg/ml rifampicin was used as positive control and 0.05 ml of DMSO was used as negative control. Antimicrobial activity was screened by adding 0.05 ml both test and standard solution to each cavity of the plate (set-1) by using micropipette. This method was performed for two more sets (Set-2, Set-3). All the plates were kept for 1 to 4 hours at room temperature and incubate them for about 18 hours at the temperature. After incubation the bacterial inhibition zone were measured in diameter with cavity from the average of three plates.

**RESULTS AND DISCUSSION**

Many naturally-occurring compounds found in plants have been shown to possess antimicrobial functions and could thus serve as a source of traditional drugs (Kim et al., 1995). **Table 2** shows the antibacterial activities of ethanolic extract of flowers against various microbial strains, with respect to various concentrations (μg/ml). The inhibition zones of test concentrations were compared with the standard concentration of rifampicin (50μg/ml) by using Tukey-Kramer multiple comparisons test. **Fig. 1, 2, 3, 4, 5, 6 and 7** shows that significant results (p<0.001 to 0.05) when compared the test concentrations versus standard drug. In the case of *Staphylococcus aureus* NCIM 2079, at test concentration (50μg/ml) versus standard drug (50μg/ml) showed insignificant (p>0.05). The antibacterial activities observed could be due to the presence of secondary metabolites. Some other reports are also reported that, various parts of this plant shows that antimicrobial activities (Kawo et al., 2009, Kareem, 2008, Bhaskar, 2000). Based the preliminary phytochemical screening of ethanolic extract of flowers of this plant, if properly screened by using additional solvents, could yield new antimicrobial drugs. Further research is therefore recommended to isolate, purify and characterize these chemical constituents.

**CONCLUSION**

As per our knowledge Ethanolic extract of flowers of *Calotropis procera* showed the antibacterial action in dose dependent on different pathogenic stains. Further studies are needed for confirmation of antibacterial action by isolating pure chemical components and also indentify which compound is responsible for antibacterial action of *Calotropis procera* flowers.

**Table 1:** Phytochemical characteristics of ethanolic extract of *Calotropis procera* flowers

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Ethanolic (70%) extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing sugar</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
</tr>
<tr>
<td>Fats</td>
<td>+</td>
</tr>
<tr>
<td>Resins</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
</tbody>
</table>

**Key:** + = Present; - = Absent.

**Table 2:** Shows the inhibition zone diameters of various concentration of ethanolic extract versus standard concentration of rifampicin

<table>
<thead>
<tr>
<th>Type of strain</th>
<th>Zone diameters (mm) with respect to Conc. of the ethanolic extract</th>
<th>Conc of Rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5μg/ml 10μg/ml 20μg/ml 30μg/ml 50μg/ml Control</td>
<td>50μg/ml</td>
</tr>
<tr>
<td>B.S.</td>
<td>10.3 ± 0.2236 12.3 ± 0.2236 13.3 ± 0.2236 14.3 ± 0.2236 15.3 ± 0.5143</td>
<td>17.3 ± 0.6708</td>
</tr>
<tr>
<td>B.P.</td>
<td>12 ± 0.4472 13.3 ± 0.2236 14.3 ± 0.2236 15 ± 0.2236 15.6 ± 0.6708</td>
<td>19.6 ± 0.6708</td>
</tr>
<tr>
<td>M.L.</td>
<td>9.6 ± 0.2236 11.6 ± 0.6708 13.3 ± 0.2236 14.6 ± 0.4919 15.6 ± 0.4919</td>
<td>20.6 ± 0.7603</td>
</tr>
<tr>
<td>P.A.</td>
<td>10.3 ± 0.5000 11 ± 1.000 13.3 ± 0.5000 14.6 ± 1.100 16 ± 1.000</td>
<td>21 ± 1.500</td>
</tr>
<tr>
<td>S.A.</td>
<td>10.3 ± 0.2236 11.3 ± 0.4472 11.6 ± 0.2236 13 ± 0.000 18.3 ± 0.2236</td>
<td>19 ± 0.4472</td>
</tr>
<tr>
<td>E.C.</td>
<td>10.3 ± 0.6708 12.6 ± 0.4919 14.6 ± 0.4919 14.6 ± 0.2236 17.3 ± 0.6708</td>
<td>20.3 ± 1.029</td>
</tr>
<tr>
<td>P.V.</td>
<td>10.6 ± 0.2236 12.3 ± 0.6708 14 ± 0.4472 16 ± 0.4472 18.3 ± 0.6708</td>
<td>23 ± 0.4472</td>
</tr>
</tbody>
</table>
The valves of each concentration of mean ± standard error by mean (S.E.M) of three replicates, Standard drug rifampicin 50μg/ml, and Ethanolic extract of flower of C.Procerea concentration (5μg/ml, 10μg/ml, 20μg/ml, 30μg/ml, and 50μg/ml). Control: Dimethylsulphoxide (DMSO). B.S: Bacillus subtilis NCIM 2063, B.P: Bacillus pumilis NCIM 2327, M.L: Micrococcus lutes NCIM 2871, P.A: Pseudomonas aeruginosa NCIM 2037, S.A: Staphylococcus aureus NCIM 2079, E.C: Escherichia coli NCIM 2067, P.V: Proteus vulgaris NCIM 2027.

Fig 1: Ethanolic extract of flower of C.Procerea shows concentration (μg/ml) versus inhibition zone diameter (mm) against Bacillus subtilis NCIM 2063. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentrations, a=5μg/ml, b=10μg/ml, c=20μg/ml, d=30μg/ml, and e=50μg/ml versus s=50μg/ml concentration of standard. The test concentration of a, b, c, d, and e are p<0.001.

Fig 2: Ethanolic extract of flower of C.Procerea shows concentration (μg/ml) versus inhibition zone diameter (mm) against Bacillus pumilis NCIM 2327. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentrations, a=5μg/ml, b=10μg/ml, c=20μg/ml, d=30μg/ml, and e=50μg/ml versus s=50μg/ml concentration of standard. The test concentration of a, b, c, and e are p<0.001.
Fig 3: Ethanolic extract of flower of *C.Procerea* shows concentration (μg/ml) versus inhibition zone diameter (mm) against *Micrococcus lutes* NCIM 2871. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentration, a = 5μg/ml, b = 10μg/ml, c = 20μg/ml, 30μg/ml, and e = 50μg/ml versus s = 50μg/ml concentration of standard. The test concentration of a, b, c, d, and e are p < 0.001.

![Fig 3](image)

Fig 4: Ethanolic extract of flower of *C.Procerea* shows concentration (μg/ml) versus inhibition zone diameter (mm) against *Pseudomonas aeruginosa* NCIM 2037. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentration, a = 5μg/ml, b = 10μg/ml, c = 20μg/ml, d = 30μg/ml, and e = 50μg/ml versus s = 50μg/ml concentration of standard. The test concentration of a, b, c, d, are p < 0.001 and e is p < 0.05.

![Fig 4](image)

Fig 5: Ethanolic extract of flower of *C.Procerea* shows concentration (μg/ml) versus inhibition zone diameter (mm) against *Staphylococcus aureus* NCIM 2079. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentration, a = 5μg/ml, b = 10μg/ml, c = 20μg/ml, d = 30μg/ml, and e = 50μg/ml versus s = 50μg/ml concentration of standard. The test concentration of a, b, c, d, are p < 0.001 and e is p < 0.05.

![Fig 5](image)
Fig 6: Ethanolic extract of flower of C. Procera shows concentration (μg/ml) versus inhibition zone diameter (mm) against Escherichia coli NCIM 2067. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentration, a=5μg/ml, b=10μg/ml, c=20μg/ml, d=30μg/ml, and e=50μg/ml versus s, 50μg/ml concentration of standard. The test concentration of a, b, c, d, and e are p<0.001 and e is p<0.05.

Fig 7: Ethanolic extract of flower of C. Procera shows concentration (μg/ml) versus inhibition zone diameter (mm) against Proteus vulgaris NCIM 2027. Tukey-Kramer Multiple Comparisons test shows the comparison of test concentration, a=5μg/ml, b=10μg/ml, c=20μg/ml, d=30μg/ml, and e=50μg/ml versus s, 50μg/ml concentration of standard. The test concentration of a, b, c, d, and e are p<0.001.

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